American Journal of Biology and Natural Sciences
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Ecological Dynamics of Mycobacterium marinum in Aquatic Environments: Interplay of Environmental Parameters and Bacterial Growth Patterns

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Received: 2024, 15, Feb
Accepted: 2025, 21, Mar
Published: 2025, 08, Apr

Annotation: This study delves into the intricate ecological dynamics of Mycobacterium marinum and Mycobacterium marinam in aquatic environments, exploring the synergistic interplay between environmental parameters and the patterns of bacterial growth. By integrating field sampling techniques, physicochemical analyses, and controlled laboratory experiments, this study aims to unveil the complex relationships between temperature, pH, dissolved oxygen levels, electrical conductivity, and the survival strategies of these mycobacterial species. Anticipated results are expected to shed light on the adaptive responses of M. marinum and M. marinam to varying environmental conditions, providing valuable insights into their ecological niches and potential implications for ecosystem health and public well-being.

Keywords: Mycobacterium marinum, Mycobacterium marinam, aquatic environments, environmental parameters, bacterial growth patterns, ecological

dynamics, physicochemical analyses, adaptive responses.

Introduction

Ciliates, protozoans characterized by hair-like cilia (Gao et al., 2016), are diverse eukaryotic microorganisms with varied morphologies (Chen et al., 2017). Estimates suggest 4,000–4,500 described free-living species, with total species projected up to 40,000 (Gao et al., 2017; Foissner & Hawksworth, 2009). Their unique traits make them valuable models in molecular biology, ecology, genetics, and evolutionary studies (Hausmann & Bradbury, 1996; Lynn, 2008).The phylum Ciliophora is classified using two primary systems: one by Corliss (1979), based on morphology, and another by Lynn (2008), emphasizing ultrastructure and SSU rRNA gene sequences, which align at the class level but differ at lower taxonomic ranks (Gao et al., 2016). Lynn's system categorizes ciliates into 57 orders and 300 families. Adl et al. (2012) further divides Ciliophora into two subphyla: Postodesmatophora (classes Karyorelictea and Heterotrichea) and Intramacronucleata, which splits into SAL (includes Lamellicorticata and Spirotrichea, covering Litostomatea and Armophorea) and CONThreeP (encompassing Plagiopylea, Phyllopharyngea, Colpodea, Nassophorea, Oligohymenophorea, and Prostomatea). Some SAL-associated genera like *Phacodinium*, *Cariacothrix*, and *Mesodinium* remain evolutionarily ambiguous (Adl et al., 2012; Chen et al., 2018).Furthermore,the classification of *Paramecium* has evolved over time. First described as "Paramecium" by John Hill in 1752, the genus underwent taxonomic revisions, including Müller's naming of *P. aurelia* in 1773 and Dujardin's later distinction of *P. caudatum* (1814). Woodruff (1921) standardized the genus name to *Paramecium* (from "Paramacium") and divided it into two morphological groups: the cigar-shaped "Aurelia" group (*P. aurelia*, *P. caudatum*, *P. multimicronucleata*) and the flattened "Bursaria" group (*P. bursaria*, *P. calkinsi*, *P. trichium*, *P. putrinum*). Early classifications also separated species like *P. versutum* under the genus *Bursaria*, reflecting ongoing refinements in taxonomy based on morphology.Modern classification of *Paramecium* spp. recognizes 18 species grouped into five subgenera (*Chloroparamecium*, *Cypriostomum*, *Viridoparamecium*, *Paramecium*, and *Helianter*) based on molecular markers and morphology, with *P. bursaria* and *P. chlorlligrum* uniquely representing *Chloroparamecium* and *Viridoparamecium*, respectively (Lanzoni et al., 2016). Ciliates exhibit vast size diversity (10 µm to 4 mm), exemplified by *Spirostomum ambiguum*, and possess motile cilia for locomotion and feeding (Lynn, 2008; Esteban et al., 2009; Gao et al., 2016, 2017). Ecologically, they inhabit diverse environments (freshwater, marine, soil) and function as heterotrophs, consuming bacteria, protozoa, and phytoplankton (Foissner & Hawksworth, 2009; Finlay & Esteban, 1998; Harrison, 1995). Their behavior, including feeding, movement, and reproduction, is influenced by environmental factors like temperature and food availability (Nche-Fambo et al., 2016; Pineda et al., 2019). Asexual reproduction occurs via fission, budding, or strobilation, while conjugation facilitates genetic exchange without increasing offspring numbers, with isogamontic or anisogamontic pairing observed in species like *Paramecium* and sessile peritrichs (Lynn, 2008; Berger, 1986; Raikov, 1972; Finley, 1943; Somasundaram et al., 2018).Ciliates, a diverse group of protists, present challenges in identification due to morphological similarities among species, necessitating staining techniques (Duff et al., 2008; Schmidt et al., 2006) or molecular approaches like DNA-based methods and phylogenetic analyses (Wang et al., 2015; Zhang et al., 2014). Their ecological roles are multifaceted: they contribute to nutrient cycling, act as decomposers, serve as bioindicators for water quality (Foissner, 1992; Payne, 2013; Xu et al., 2015), and function as predators in microbial loops (Azam et al., 1983). In laboratory settings, culturing ciliates like *Paramecium* requires specific media, with B-5 Basal medium proving optimal compared to Hay infusion or Alfalfa (Lewis & Collins, 2010; Matsushima et al., 2022). Environmental factors

such as temperature, pH, salinity, and nutrient availability significantly influence ciliate communities, affecting their growth and distribution (Dias et al., 2008; Pedersen & Hansen, 2003; Montagnes & Weisse, 2000). For instance, *Paramecium bursaria* thrives at neutral pH and moderate temperatures but exhibits resilience under alkaline conditions (Fels & Kaltz, 2006; Warren et al., 2017). Recent studies highlight advancements in characterizing ciliate diversity through molecular techniques, enhancing understanding of their ecological interactions and adaptability (Gao et al., 2016; Warren et al., 2017). This research underscores the importance of standardized culturing protocols, multi-factorial growth assessments (cell count, optical density, dry weight), and environmental parameter optimization to advance ciliate studies in both ecological and laboratory contexts.

Materials & methods

Sample Site Selection

The research sample for this study was obtained from the River Jehlum at various locations. The river runs through different areas, providing a diverse range of water samples for analysis.

Sample Collection

The sampling process involved two phases: the first phase occurred on 3rd December 2021, and the second phase on 7th April 2022. Samples were collected from the riverbank at three different times of the day: morning (M1, M2, M3 at 9:00 am), noon (N1, N2, N3 at 12:00 pm), and evening (E1, E2, E3 at 3:00 pm). Each sample was collected in clean sterile bottles, with environmental parameters such as temperature, pH, dissolved oxygen, humidity, precipitation, and wind speed being recorded during each sampling event. Furthermore, the water analysis report encompassed various environmental variables like electrical conductivity, total dissolved solids (TDS), residual sodium carbonates (RSC), and sodium adsorption ratio, with analysis conducted at the agricultural water analysis lab in Sargodha, as per Loureiro, Junior, & Abreu.

Sample Preparation

Following collection, the samples were placed in sterile capped plastic bottles and enriched with green leaves, dried leaves, ceramics, and soil from the area. The mixture was then agitated and left undisturbed for two months. Throughout this period, continuous microscopic observations were conducted. Initially, a bacterial bloom was observed, followed by an increase in ciliate populations as they fed on the bacteria. After a few weeks, the water sample cleared, leaving behind predominantly ciliates.

Growth Culturing

After one month, culturing of different ciliates commenced. By the end of two months, the culturing process had achieved 70% completion. Rice grain powder was introduced to further stimulate ciliate growth. Regular microscopic observations were carried out during the culturing stages, with ciliate counts documented after three months, as described by Thorp in 2015.

Identification

The identification of ciliates was accomplished using a taxonomic classification key. Morphological characteristics were examined through microscopy and staining. A detailed observation process involved placing a sample droplet on a deep well slide and examining it under varying microscope resolutions (4X, 10X, and 40X). Additionally, a fixed slide was prepared using formalin and methylene blue staining for enhanced visualization under a microscope, following the methodology outlined by Abraham et al. in 2019.

Isolation

Ciliates were isolated from the sample using a micropipette under a microscope. Post-isolation, the ciliates were transferred to a media broth to facilitate further culturing, as described by Pedroso Dias, D'avila, Wieloch, & D'Agosto in 2008.

Media Preparation

For the culturing of ciliates, three distinct media types—Hay infusion, alfalfa medium, and B-5 basal media broth—were prepared according to their specific compositions and requirements. These media types were crucial for sustaining and fostering the growth of ciliates throughout the study.

Hay infusion

Hay infusion consists of the following materials.

Composition:

Hay infusion	
Timothy Hay	10g
NaOH	2 drops
Distilled Water	1 liter

To make hay infusion (medium) 10 g of timothy Hay is added into the 1 liter of distilled water and sterilized for 30 minutes. After sterilization, 2 drops of NaOH were added in the medium (Rao & Hussain, 2012).

Alfalfa Medium

Alfalfa medium is formed by the following composition.

Composition:

Alfalfa Medium		
Alfalfa		1 stalk
Chalkey's solution	NaCl	1g
	KCl	0.04g
	$CaCl_2$	0.06g
Distilled Water		1 liter

To make alfalfa medium alfalfa is boiled with Chalkey's solution for each liter. The alfalfa was allowed to absorb and form sediment on the bottom. Sediments are further used as food for ciliates (Hummer Jr, 1993).

B-5 Basal Medium

B-5 Basal Medium was developed by adding the following chemicals in 1000 ml of distilled water.

Composition:

B-5 Basal Medium	
B-5 Basal Medium	3.21 g/l
Distilled water	1:1000

B-5 basal media was formed by adding 3.21 g in the 1L of distilled water.

Culturing

Hay infusion

The medium was sterilized and poured into the flask when cooled. After this, 200 ml of medium was placed in the flask and 200 ml of ciliates culture was added in the medium. Now the medium was left for 2 weeks for highest growth (Rao & Hussain, 2012).Hay infusion is the most suitable and essential medium which is used to culture ciliates. It provides the nearest environment like

their own habitat. It stimulates the growth of bacteria which is used by ciliates as a food. Now ciliates grow normally in this medium (Rao & Hussain, 2012).

Alfalfa medium

The 200 ml of prepared (boiled) alfalfa medium was added into the flask and was cooled. Now 200 ml of ciliates culture was added to this flask. After this, 5 g of chopped alfalfa was added to the flask. Now 10 boiled wheat seeds were added to the flask. This medium was left for 3 weeks to reach the peak of the growth (Hummer Jr, 1993). Alfalfa medium is an excellent medium that is used to grow a large number of ciliates, especially *Paramecium*. The chopped alfalfa sediment will be served as a source of food for ciliates (Hummer Jr, 1993).

B-5 Basal Medium

B-5 basal media is used to culture the plant cells. Ciliates feed on these plant cells and ultimately grow in the medium. For the preparation of B-5 Basal Medium, 3.21 g of B-5 basal media was added in 1000 ml of distilled water. The sample was mixed well and autoclaved. Now chopped green leaves were added in the media. In the prepared B-5 basal media, 100 ml of inoculum was added.

Effect of temperature and pH on the growth of ciliates

Nine samples of B-5 basal media were prepared to investigate ciliate growth under varying temperatures and pH levels. Temperature variations included incubator (30-40°C), room (20-30°C), and low (10-20°C) temperatures, while pH levels ranged from acidic (5-6) to basic (8-9). Each sample was subjected to combinations like low temperature with acidic pH, room temperature with neutral pH, and incubator temperature with basic pH to study their effects on ciliate growth (Jafari & Alavi, 2010).

After culturing, all samples underwent centrifugation at 7000 rpm for five minutes, with each sample centrifuged three times. The resulting pellets were of varying sizes, indicating different growth rates (Beisson et al., 2010). Dry weights of the samples were measured to quantify ciliate growth.

Spectrophotometry at a 650 nm wavelength was used to measure the optical density of each sample in glass cuvettes. This optical density, assessed three times for accuracy, served as a metric for understanding ciliate growth levels (Glud & Fenchel, 1999; Schreiber & Brink, 1989).

Materials and Methods:

Sample Collection and Preparation:

Water samples were collected from River Jehlum at designated locations consistent with the study by Jafari and Alavi (2010). A total of three sampling stations were selected to represent distinct environmental conditions. Each sample was carefully collected and transported to the laboratory for further analysis.

Physicochemical Analysis:

The collected water samples were analyzed for various physicochemical parameters including temperature, pH, dissolved oxygen, and electrical conductivity. The procedures followed were in accordance with the methods described by Dias et al. (2008) for water quality analysis. Temperature measurements were recorded using a calibrated thermometer, pH values were determined with a pH meter, dissolved oxygen levels were quantified using appropriate probes, and electrical conductivity was measured using a conductivity meter.

Mycobacterium marinum cultures were established in the laboratory under controlled conditions mimicking the temperature and pH ranges observed in the natural water bodies. The cultures were maintained at temperatures ranging from 10.10°C to 29.7°C to simulate seasonal variations as

described in the study by Jafari and Alavi (2010). The pH of the culture media was adjusted within the optimal range of 7.0-7.6 as recommended by Littleford (1960) for enhanced growth of ciliates.

DataAnalysis:

The obtained data on physicochemical parameters and their effects on *Mycobacterium marinum* growth were analyzed using appropriate statistical methods to determine correlations and potential impacts on the bacterial population dynamics.

Results

Identification of Paramecium bursaria

During the analysis of samples from Kalar Kahar Lake, the species identified across all samples was Paramecium bursaria. The identification process involved cross-referencing the species with three classification keys, utilizing morphological features for accurate identification. The classification keys used were authorized by the World Health Organization (Bick & Organization, 1972), John O. Corliss (John O. Corliss, 2016), and Wilhelm Foissner and H. Berger (Wilhelm Foissner & Berger, 1996).

The species, Paramecium bursaria, was confirmed through microscopic observation, as depicted in Figure 3.1.4a.

Identified species

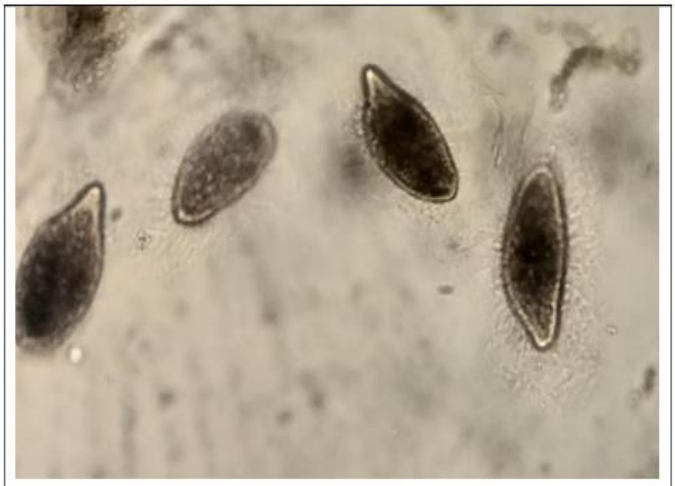

Figure 3.1 a*: Paramecium bursaria* was identified after microscopy

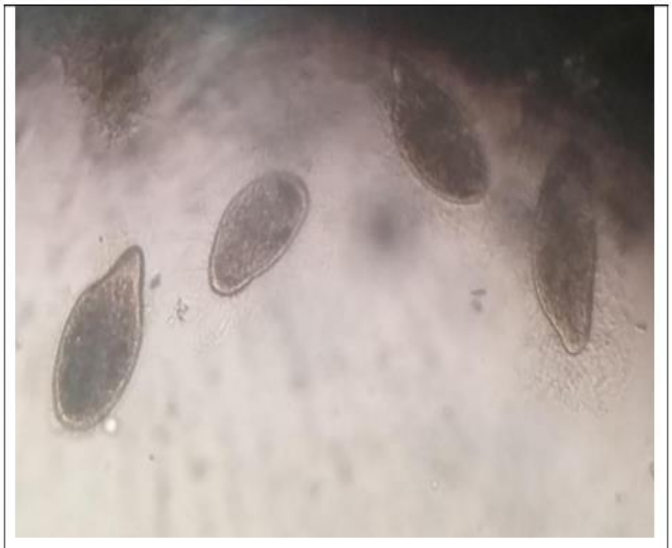

Figure 3.1 b: Paramecium bursaria was identified after microscopy

Environmental parameters of the samples collected from River Jehlum

Environmental parameters of the River Jehlum were meticulously evaluated at all stations during two sampling phases: 3rd December 2021 and 7th April 2022. The parameters examined included temperature, pH, dissolved oxygen, wind speed, humidity, precipitation, carbonates, bicarbonates, calcium + magnesium, sodium, chloride, sodium absorption ratio, residual sodium carbonates (RSC), and electrical conductivity.During both sampling phases, the on-site parameters of temperature, pH, dissolved oxygen, wind speed, humidity, and precipitation were directly measured. The comprehensive analysis of the remaining environmental parameters was conducted by the Agriculture Department of the Government of Punjab.The detailed records of these environmental parameters for both sampling phases are presented in Table 3.2.1.1 and Table 3.2.2.1 respectively.

Environmental parameters at first phase of samplings

The environmental parameters of the River Jehlum were meticulously assessed at various stations simultaneously during both sampling phases. Parameters including temperature, pH, dissolved oxygen, wind speed, humidity, precipitation, carbonates, bicarbonates, calcium + magnesium, sodium, chloride, sodium absorption ratio, residual sodium carbonates (RSC), and electrical conductivity were examined during the study.

In the first sampling phase, the temperature across all nine samples averaged approximately 14.33 ± 0.33°C. The highest temperature was recorded at noon, measuring 15.77 ± 0.44°C. Significant temperature variations were observed among morning, noon, and evening samples, with similarities noted between morning and noon samples.

pH levels ranged from 7.7 ± 0.10 to 8.23 ± 0.67, with the highest pH recorded in the noon samples. Dissolved oxygen levels at the sampling site varied from 3.37 ± 0.12 to 4.37 ± 0.12 mg/L, with the morning sample exhibiting the highest value.

Wind speeds ranged from 10.00 ± 0.58 to 14.33 ± 0.33 km/h, with significant differences observed in the morning samples. Humidity levels fluctuated between 73.33 ± 2.19 and 82.33 ± 0.33%, with significant variations noted among evening samples. Precipitation was recorded at 3 ± 0.00%.

Additional parameters such as electrical conductivity, calcium+magnesium, sodium, bicarbonates, chloride, and sodium absorption ratio were analyzed. Electrical conductivity ranged from 2100 ± 5.77 to 2156.67 ± 3.33 µS/cm, with the highest values in the noon samples. Calcium+magnesium levels varied from 9.20 ± 0.58 to 9.27 ± 0.03 meq L-1, with no significant differences observed.

Sodium levels ranged from 11.87 ± 0.88 to 12.30 ± 0.06 meq L-1, with the highest values in the noon samples. Bicarbonate and chloride levels showed minimal variation with no significant differences. Sodium absorption ratio values fluctuated between 5.50 ± 0.58 and 5.73 ± 0.33, with significant differences noted between noon and evening samples.

No significant differences were observed in the residual sodium carbonates (RSC) values across the samples. The comprehensive dataset of all these environmental parameters is available in Table 3.1.1.1 for further reference.

Table 3.2.1.1: Parameters of the 1st Samples at the site of sample collection dated 03-12-21.

Parameters	Morning	Noon	Evening
Temperature	13.67 ± 0.33^{b}	15.17 ± 0.44^{a}	14.33 ± 0.33^{ab}
pH	7.70 ± 0.100^{b}	8.23 ± 0.67^{a}	7.83 ± 0.33^{b}
Dissolved oxygen	$4.37\pm0.12_{a}$	3.37 ± 0.12^{b}	4.20 ± 0.58^{a}
Wind speed	$14.33\pm0.33_{a}$	10.00 ± 0.58^{b}	11.33 ± 0.88^{b}
Humidity	80.33 ± 0.882^{a}	82.33 ± 0.33^{a}	73.33 ± 2.19^{b}
Precipitation	3.00±0.00	3.00±0.00	3.00±0.00

Electrical conductivity	2113.33±8.82[b]	2156.67±3.33[a]	2110.00±5.77[b]
Calcium Magnesium salt levels	9.20±0.58[a]	9.27±0.03[a]	9.23±0.33[a]
Sodium	11.93±0.15[b]	12.30±0.06[a]	11.87±0.88[b]
Bicarbonate	8.13±0.07[a]	8.20±0.00[a]	8.13±0.67[a]
Chloride	10.07±0.03[a]	9.97±0.67[a]	9.97±0.33[a]
Sodium absorption ratio	5.53±0.09[ab]	5.73±0.33[a]	5.50±0.58[b]
Residual sodium carbonates	0.00	0.00	0.00
N	9	9	9

Environmental parameters at second phase of sampling

In the second phase of sampling at River Jehlum, the temperature of all samples ranged from 29 ± 0.58 to 37 ± 0.58°C. Evening temperatures showed significant differences compared to morning and noon samples. The pH values ranged from 8.0 ± 0.06 to 8.13 ± 0.89, with the highest pH recorded in the noon sample. There were no significant differences in pH among the samples.

Dissolved oxygen levels ranged from 4.43 ± 0.09 to 4.63 ± 0.33 mg/L, with the highest reading in the noon sample. Wind speeds at the sampling site varied from 4.33 ± 0.33 to 11.67 ± 0.33 km/h, showing significant differences between samples. Humidity levels ranged from 61.0 ± 01.00 to 63.67 ± 0.89%, with significant differences noted.

Precipitation levels were noted to be 0% in all samples during this phase of sampling.

Additional parameters analyzed from the Government of Punjab institute included electrical conductivity, calcium+magnesium, sodium, bicarbonates, chloride, and sodium absorption ratio. Electrical conductivity ranged from 2406 ± 3.33 to 2766 ± 366.67 µS/cm, with the highest value observed in a morning sample. Calcium+magnesium levels ranged from 9.20 ± 0.10 to 9.30 ± 0.00 meq L-1, with no significant differences.

Sodium levels varied from 14.53 ± 1.67 to 15.40 ± 0.53 meq L-1, with the highest value in an evening sample. Bicarbonate levels ranged from 8.27 ± 0.13 to 8.40 ± 0.00 meq L-1, with no significant differences observed. Chloride values fluctuated from 12.97 ± 0.09 to 13.23 ± 0.03 meq L-1, with the highest value recorded at noon.

Sodium absorption ratio values ranged from 6.70 ± 0.10 to 7.13 ± 0.30, with the highest value in the evening sample and no significant differences observed. RSC values were not detected in any of the samples analyzed. The detailed dataset of these parameters can be found in Table 3.2.2.1 for further reference.

Parameters of the 2nd Samples at the site of sample collection dated 7-04-22.

Parameters	Morning	Noon	Evening
Temperature	35.33±0.33[a]	37.00±0.58[a]	29.00±0.58[b]
pH	8.03±0.33[a]	8.13±0.89[a]	8. 00±0.06[a]
Dissolved oxygen	4.53±0.88[a]	4.63±0.33[a]	4.43±0.09[a]
Wind speed	4.33±0.33[c]	8.33±0.33[b]	11.67±0.33[a]
Humidity	61.00±1.00[a]	63.67±0.89[a]	61.00±1.53[a]
Precipitation	0.00±0.00	0.00±0.00	0.00±0.00
Electrical conductivity	2766.67±366.67[a]	2406.67±3.33[a]	2460.00±43.59[a]
Calcium Magnesium salt levels	9.30±0.00[a]	9.27±0.03[a]	9.20±0.10[a]
Sodium	14.53±1.67[b]	14.80±0.06[a]	15.40±0.53[a]
Bicarbonates	8.33±0.07[a]	8.40±0.00[a]	8.27±0.13[a]
Chloride	13.03±0.19[a]	13.23±0.03[a]	12.97±0.09[a]

Sodium absorption ratio	6.70±0.10[a]	6.83±0.03[a]	7.13± 0.30[a]
Residual sodium carbonates	0.00	0.00	0.00
N	9	9	9

Media difference based on ciliates cell count, Optical density and pellet size

In the study conducted at River Jehlum, the growth of ciliates was carefully examined in three different media: Hay infusion, Alfalfa medium, and B-5 basal medium. Following the culturing process, the ciliate cell count, optical density, and pellet size values were assessed to evaluate the growth of the samples.

For the Hay infusion medium, the ciliate cell count, optical density, and dry weight of all samples were determined in triplicates. The ciliate cell count was conducted by placing a 25 µl drop of the sample on a microscopic slide. Over the course of four weeks, fluctuations in ciliate cell count were observed. In the first week, the count was 8.33 ± 0.53, increasing to 17.78 ± 0.09 in the second week, notably in sample N2. However, growth decreased to 11.22 ± 0.74 in sample M2 in the third week due to food and dissolved oxygen shortages. By the fourth week, the count significantly dropped to 3.33 ± 0.37 in samples M2 and E1.

The optical density values were also monitored weekly. In the first week, values ranged from 0.297 ± 0.01 for sample M2 to 0.326 for sample N2. The optical density peaked in the second week at 0.4116 ± 0.02 for sample N2 but decreased to 0.3378 ± 0.02 in the third week (sample E3) due to resource shortages. By the fourth week, values decreased further to 0.1537 ± 0.01 in sample M2.

The dry weights of the samples were calculated through centrifugation. In the first week, all samples had a dry weight of 0.03 ± 0.00, with the highest value in sample N2. By the second week, the dry weight increased to 0.04 ± 0.00, displaying the highest growth. However, in the third week, values decreased to 0.03 ± 0.00, and by the fourth week, they further dropped to 0.01 ± 0.00, notably in samples M2 and N2. Significant differences in dry weights were observed among the samples throughout the four-week period.

Table 3.3.1.1: Growth trend estimation by Ciliate cell count, optical density and dry weight of Samples cultured in Hay infusion

Growth in Hay infusion	Week 1	Week 2	Week 3	Week 4
Ciliates cell count (N=9)	8.33±0.53[c]	15.78±0.97[a]	11.22±0.74[b]	3.33±0.37[d]
Optical density (N=9)	0.2697±0.01[c]	0.4116±0.02[a]	0.3378±0.02[b]	0.1537±0.01[d]
Dry weight (N=9)	0.0272±0.00[c]	0.0421±0.00[a]	0.0322±0.00[b]	0.0158±0.00[d]

Alfalfa medium

In alfalfa medium, the ciliates cell count was given in all four weeks of the culturing. There was not much growth of ciliates in this medium. In the first week of culturing, the ciliates cell count ranged from 0.67±0.17. This means that only one ciliate was observed in 25 µl of the sample. One ciliate was found in some samples including M1, M2, N1, N2, E1, and E3. In the second week, ciliate count was only in sample N1 and E3 and reached up-to 0.56±0.29. In other samples ciliates were found dead except M2. In week three and four, no live growth was observed. Week 3 and 4 showed a significant difference against week 1 and 2. All these ciliates cell counts were given in table 3.3.2.1.

Table 3.3.2.1: Growth trends estimation by ciliate cell count of Samples cultured in Alfalfa medium.

Growth in Alfalfa medium	Week 1	Week 2	Week 3	Week 4
Ciliates cell count (N=9)	0.67±0.17[a]	0.56±0.29[a]	0.00±0.00[b]	0.00±0.00[b]

B-5 Basal Medium

In B-5 basal medium the growth was observed by ciliates cell count, optical density and dry weight of these samples. The values of ciliates cell count were 499.67±11.928 in the 1st week, 755.33±31.938 in the 2nd week, 306.22±24.216 in the 3rd week and 34.33±4.308 in the 4th week. All the values showed significant differences between the weeks. The values of optical density were 9.962±0.306 in the 1st week of the culturing, 15.369±0.905 in the 2nd week, 6.229±0.486 in the 3rd week and 0.788±0.080 in the 4th week. There was a significant difference present between the samples of four weeks. The values of dry of the samples were 1.934±0.885 in the 1st week of the culturing, 1.575±0.101 in the 2nd week, 0.631±0.051in the 3rd week and 0.386±0.1154 in the 4th week of the culturing. The 4th week showed a significant difference from the other three weeks. The 2nd and 3rd weeks showed resemblance with 1st week and 4th week. All these values are given in table 3.3.3.1.

Table 3.3.3.1: Growth trend estimation by ciliate cell count, optical density and dry weight of the samples cultured in B-5 Basal medium

Growth in B-5 Basal medium	Week 1	Week 2	Week 3	Week 4
Ciliates cell count (N=9)	499.67±11.928[b]	775.33±31.938[a]	306.22±24.216[c]	34.33±4.308[d]
Optical density (N=9)	9.962±0.306[b]	15.369±0.905[a]	6.229±0.486[c]	0.788±0.080[d]
Dry weight (N=9)	1.934±0.885[a]	1.575±0.101[ab]	0.631±0.051[ab]	0.386±0.114[b]

Effect of temperature and pH on the growth of *p. bursaria*

In the B-5 basal medium, there were nine samples named S1-S9 respectively that were used to check the effect of temperature and pH. To check the effect of temperature and pH, different variations were made for temperature and pH. These variations were made on the basis of 3 different temperatures, and three different pH. Total these nine variations were made. These different temperatures were used including normal room temperature (20-30°C), low temperature at (10-20°C) and incubator temperature at (30-40°C). Three different pH were used including acidic pH (5-6), neutral pH (7) and basic pH (8-9). These all nine temperatures and pH parameters were checked on all these samples. These entire nine samples were counted and their count was observed and noted. The Optical density and the Dry weights values were also noted. The values of ciliate cell count of all nine samples were given in table 3.4.1.1, optical density in table 3.4.1.2, and dry weight in table 3.4.1.3. All these three procedures provided the proof of the growth of ciliates culturing.

Ciliates cell count values

In the environmental study conducted at River Jehlum, ciliate cell counts were examined under different temperature and pH conditions. Significant growth variations were observed across the different settings. For instance, in the low temperature range (10-20°C) and neutral pH (7), a

steady increase in ciliate cell counts was noted over the weeks, with significant differences observed between all values. In contrast, at normal room temperature (20-30°C) and acidic pH (5-6), a notable spike in cell counts was observed in the 2nd week, followed by a decrease in the 3rd and 4th weeks, with significant differences between the 1st, 2nd, and 4th weeks. Similarly, under incubator temperature conditions (30-40°C) and basic pH (8-9), a significant variance was seen between weeks, with the 2nd, 3rd, and 4th weeks displaying substantial differences in cell counts. These findings underscore the complex interplay between tempeature, pH, and ciliate growth dynamics in aquatic ecosystems.

Table 3.4.1.1: Effect of Temperature and pH estimation by ciliates cell count of sample per 25 µl of Samples cultured in B-5 Basal Medium

Temperature of the samples	pH of the samples	Ciliates cell count per 25 µl			
		Week 1	Week 2	Week 3	Week 4
Temperature (10-20)	Acidic (5-6)	6.00±0.58^{b}	11.33±1.45^{a}	7.00±2.31^{ab}	1.00±0.58^{c}
	Neutral (7)	27.67±2.40^{b}	38.33±1.76^{a}	13.67±3.71^{c}	1.33±0.33^{d}
	Basic (8-9)	9.00±1.15^{b}	14. 67±0.88^{a}	9.33±0.88^{b}	2.67±0.88^{c}
Temperature (20-30)	Acidic (5-6)	87.67±4.26^{b}	244.00±21.93^{a}	101.00±5.57^{b}	17.67±3.18^{c}
	Neutral(7)	428.33±20.54b	524.33±16.56^{a}	267.00±19.08^{c}	11.33±1.76^{d}
	Basic (8-9)	283.00±26.50b	747.33±30.19^{a}	299.00±45.90^{b}	22.33±6.96^{c}
Temperature (30-40)	Acidic (5-6)	369.33±31.54b	897.00±64.16^{a}	338.33±47.24^{b}	12.00±2.08^{c}
	Neutral (7)	532.67±36.97b	868.33±33.17^{a}	597.00±35.08^{b}	95.67±2.91^{c}
	Basic (8-9)	530.33±73.64b	894.00±91.00^{a}	335.00±67.10^{b}	42.67±15.43c

Optical density

In the study conducted at River Jehlum, the optical density values exhibited a similar trend to ciliate cell counting across different temperature and pH conditions.

In the low temperature setting, the neutral pH showed the most significant growth. In the acidic pH, growth reached 0.33 ± 0.03 in the 2nd week, compared to the peak growth of 0.84 ± 0.03 in the 2nd week in the neutral pH. Similarly, the basic pH reached a growth of 0.40 ± 0.02 in the 2nd week. The trend of maximum growth was observed in the neutral pH, followed by the basic pH, with the acidic pH showing the least growth. There were no significant differences observed between the acidic, neutral, and basic pH levels in the low temperature conditions.

At a slightly higher temperature range (10-20°C), the neutral pH demonstrated the highest growth, followed by the basic pH, and finally the acidic pH. Notably, in the acidic pH of the low temperature, a significant difference was observed in the 4th week compared to other sample values.

In the normal room temperature conditions, significant differences were noted in the growth patterns. The acidic pH showed growth of 1.84 ± 0.09 in the 1st week, 4.98 ± 0.40 in the 2nd week, 2.09 ± 0.13 in the 3rd week, and 0.44 ± 0.05 in the 4th week. The neutral pH and basic pH also displayed distinct growth patterns over the four-week period.

Under incubator temperature conditions, growth varied in the acidic, neutral, and basic pH levels. Notably, the neutral pH demonstrated the highest growth rates in the 2nd week, with significant differences observed across the weeks for each pH level. The basic pH showed a steady decrease in growth from the 2nd to the 4th week. Maximum growth was observed in the basic and neutral pH levels under incubator temperature conditions.

Table 3.4.2.1: Effect of Temperature and pH estimation by optical density of sample per 5 ml of Samples cultured in B-5 Basal Medium.

Temperature of the samples	pH of the samples	Optical density per 5 ml			
		Week 1	Week 2	Week 3	Week 4
Temperature (10-20)	Acidic (5-6)	0.22±0.01^{a}	0.33±0.03^{a}	0.24± 0.05^{a}	0.08±0.04^{b}
	Neutral (7)	0.60±0.06^{b}	0.84±0.03^{a}	0.37±0.067^{c}	0.11±0.01^{d}
	Basic (8-9)	0.28±0.02^{b}	0.40±0.02^{a}	0.29±0.02^{b}	0.14±0.02^{c}
Temperature (20-30)	Acidic (5-6)	1.84±0.09^{b}	4.98±0.40^{a}	2.09±0.13^{b}	0.44±0.05^{c}
	Neutral (7)	8.40±0.15^{b}	10.54±0.35^{a}	5.10±0.63^{c}	0.33±0.04^{d}
	Basic (8-9)	5.83±0.52^{b}	15.00±0.63^{a}	5.37±1.13^{b}	0.54±0.14^{c}
Temperature (30- 40)	Acidic (5-6)	7.20±0.82^{a}	4.98±0.40^{a}	6.84±0.97^{a}	0.63±0.29^{b}
	Neutral (7)	10.40±0.99^{b}	17.49±0.64^{a}	12.00±0.70^{b}	2.04±0.0c9^{c}
	Basic (8-9)	10.47±1.16^{b}	17.98±1.82^{a}	5.45±0.48^{c}	0.95±0.30^{d}

Dry weight

In the study conducted at River Jehlum, the dry weight values were analyzed in different temperature and pH conditions, namely low temperature, normal room temperature, and incubator conditions.

In the low temperature setting, the dry weight values varied across acidic, neutral, and basic pH levels over four weeks. Notable growth was observed in the neutral pH of low temperature conditions.

At normal room temperature, distinct patterns were observed in the dry weight values across acidic, neutral, and basic pH levels. Maximum growth was noted in the neutral and basic pH conditions.

Similarly, in the incubator temperature environment, the dry weight values fluctuated under acidic, neutral, and basic pH conditions. Notably, significant differences were observed in the growth patterns, with the maximum growth seen in the neutral pH of the incubator temperature.

Throughout these experiments, a consistent trend was observed where growth increased in the initial weeks and declined in the later stages. This decrease in growth was attributed to food and dissolved oxygen shortages as the ciliates consumed available resources over time, leading to reduced growth rates in the later weeks of the study.

Table 3.4.3.1: Effect of Temperature and pH estimation by dry weights of sample per 45 ml of Samples cultured in B-5 Basal Medium.

Temperature of the samples	pH of the samples	Dry weights per 45 ml			
		Week 1	Week 2	Week 3	Week 4
Temperature (10- 20)	Acidic (5-6)	0.02±0.00^{a}	0.03±0.00^{a}	0.02 ±0.01^{a}	0.01±0.00^{b}
	Neutral (7)	0.06±0.00^{b}	0.08±0.00^{a}	0.04±0.01^{c}	0.02±0.01^{c}
	Basic (8-9)	0.03±0.00^{b}	0.04±0.00^{a}	0.030.00^{b}	0.01±0.00^{c}
Temperature (20-30)	Acidic (5-6)	0.05±0.01^{c}	0.41±0.00^{a}	0.22±0.01^{b}	0.05±0.00^{d}
	Neutral (7)	0.75±0.06^{b}	1.31±0.30^{a}	0.47±0.06^{bc}	0.03±0.00^{c}
	Basic (8-9)	0.62±0.12^{b}	1.51±0.06^{a}	0.49±0.08^{b}	0.06±0.01^{c}
Temperature (30-40)	Acidic (5-6)	0.67±0.07^{b}	1.69±0.18^{a}	0.69±0.12^{b}	0.03±0.00^{c}
	Neutral (7)	0.95±0.07^{c}	1.69±0.07^{a}	1.16±0.05^{b}	0.20±0.01^{d}
	basic (8-9)	1.01±0.12^{b}	1.75±0.18^{a}	0.61±0.12^{b}	0.10±0.03^{c}

The growth of the ciliates was Maximum in the B-5 basal medium. The growth trend was estimated by ciliates cell count, optical density and dry weight. These three factors provided the best suitable results of ciliates culturing. The change in temperature and pH showed an effect on the growth of *Paramecium bursari*. The increase and decrease of temperature caused the death of the *Paramecium*. The low temperature (10-20) also affected the *Paramecium* sp. and it killed the *P.bursaria* and its number decreased in the sample and medium. In the normal room temperature (20-30) the growth was higher than the lower temperature. In the incubator temperature (30-40) the growth was so exponential.

The change in the pH especially in the acidic pH (5-6), the number of ciliated decreased suddenly. The neutral pH (7) was the most suitable pH for the ciliate. The increase in the pH up- to 8-9 is not dangerous for ciliates because *Paramecium* bursaria is resistant to the change in pH from neutral pH to the basic (8-9) pH.

So it is concluded that the change in temperature and pH affected the growth and number of the *Paramecium sp*

Discussion

Environmental parameters

In the study by Littleford (1960) on free-living protozoans, the crucial role of temperature in the life cycle of protozoa was highlighted. The culture temperature ranged from 65-76°F, with growth ceasing at 53°F (11.67°C) and a sharp decrease in protozoa numbers above 86°F (30°C). Maintaining a neutral pH was emphasized for optimal and rapid ciliate growth, with the recommended range being between 7.0-7.6. While light was unnecessary for most protozoa cultures, Paramecium bursaria specifically required light for growth.

In another study by Dias et al. (2008), four physiological parameters of water samples from three stations were examined. The sample temperatures ranged from 22.4°C to 23.2°C, pH values varied from 7.43 to 8.11, dissolved oxygen concentrations ranged from 3.02 to 6.39 mg/L, and electrical conductivity spanned from 78.6 to 293.0 µS/cm.

Furthermore, in the study by Jafari and Alavi (2010) focusing on plankton communities and seasonal variations, temperature fluctuations from 10.10°C in January to 29.7°C in July were noted. pH levels ranged between 7.18 and 8.12, while dissolved oxygen concentrations varied from 3.10 to 5.60 mg/L. Seasonal differences were observed in electrical conductivity and alkalinity, with phosphate levels ranging from 0.88 to 0.09 mg/L and nitrogen concentrations from 1.05 to 2.34 µg/L. These studies collectively underscore the importance of various environmental factors in influencing the growth and dynamics of aquatic organisms.

In another study by Khan, Aadil, and Khan (2011) focusing on the environmental parameters of Kalar Kahar Lake, temperature fluctuations were noted from 17 to 32°C. The pH values ranged from 6.46 to 7.98, and electrical conductivity varied between 698 and 1046 µS/cm. Chloride levels ranged from 112 to 159 mg/L, bicarbonate values were between 102 and 128 mg/L, and calcium concentrations ranged from 34 to 56 mg/L. Additionally, magnesium levels varied from 21 to 36 mg/L, sodium concentrations ranged from 29 to 45 mg/L, potassium values were between 12 and 20 mg/L, and phosphorus levels varied from 40 to 65 mg/L.

In the study by Kulaš et al. (2021) focusing on the environmental parameters of the Krka river, temperature values ranged from 10.3 to 15.4°C. Dissolved oxygen concentrations were between 9.5 and 10.26 mg/L, and pH values at sample sites ranged from 7.75 to 8.35. Electrical conductivity values ranged from 391 to 690 µS/cm. These studies provide valuable insights into the diverse physical and chemical parameters of different water bodies, shedding light on the environmental conditions and variations in aquatic ecosystems.

In a recent study conducted by Acha, William, and Gideon (2022) along River Jhelum, various environmental parameters were monitored. The temperature of the water samples fell within the

range of 24.5-26.3°C, while dissolved oxygen levels varied from 42.5% to 80.50%. Electrical conductivity measurements ranged from 25.67 to 761.75 µS/cm, and the pH values fluctuated between 5.8 and 7.2. Turbidity values were recorded between 6.0 and 116.7 FTU. The suspended solids content ranged from 4.5 to 91.0 mg/L, with a mean value of 25.37±18.21 mg/L. Nitrates were found to have a mean value of 1.70±0.88 mg/L, while nitrite levels ranged from 0.009 to 0.170 mg/L. Ammonium concentrations in the samples varied from 0.02 to 0.33 mg/L. This comprehensive analysis provides valuable insights into the water quality parameters along the River Jehlum, crucial for understanding the ecosystem dynamics and potential environmental implications.

Importance of culturing ciliates especially *Paramecium bursaria*

The importance of cultivating *P.bursaria* is given as follows.

1. The most important of the ciliate is that the *Paramecium* has eye-catching features for research. *Paramecium* is used in research many times.

a) *Paramecium* can easily be obtained from nature, investigators and commercial sources.

b) It can be isolated and cultured from the simplest way to the toughest method.

c) *Paramecium* has a small size that can't be seen from the naked eye. So it can be cultured in large amount in a small place easily

d) It can be handled easily, so we can observe all the parts of that *Paramecium*.

e) *Paramecium* can reproduce very fast so it can be cultured in small generation time varying from 4 hours to 8 hours. The generation time depends on the species.

f) The *Paramecium* showed the most differentiated cells. These differentiated cells, especially cortex providing the stability and chances for the development analyses.

g) Reproduction (conjugation) can be initiated by the chemicals (Sonneborn, 1970).

2. *Paramecium bursaria* contains symbionts like algae which has importance in biology for the wide variety of expanded ranges of biological studies like researches (Sonneborn, 1970).

3. The symbiosis of *Paramecium bursaria* with green algae gives the conspicuous green color to the *P.bursaria* which gives the chance for constant and natural marker (Jennings, 1938; Sonneborn, 1970).

4. Its physical appearance attracts students to study on them. It helps the student to examine the living protozoa which was just seen only in the textbook (Lewis & Collins, 2010).

5. Paramecia are used for the feeding of fry fish like zebrafish and medaka in the lab of embryology (Lewis & Collins, 2010).

6. *Paramecium bursaria* are the green Paramecia which show symbiosis with algae. Symbiotic algae only present in the *P.bursaria*. *P.bursaria* contains hundreds of algae inside it. To understand the relationship of symbiosis, *Paramecium Bursaria* are the common and easiest example to observe and study (Matsushima et al., 2022).

Natural conditions indicate the suitability of conditions supporting the survival of *Paramecium*, is it true?

In this study conducted by Khan et al. (2011), he observed the environmental parameters of the Kalar Kahar Lake. The temperature observed at the Lake ranged from 17-32°C. The effect of different temperatures was checked on the growth of *Paramecium bursaria*. Three different temperatures were used including low temperature (10-20), normal room temperature (20-30) and incubator temperature (30-40). At the low temperature, the growth of *P.bursaria* in the B-5 Basal medium was reached at 36.33±1.76. This was the maximum growth achieved in the low temperature and it belonged to the neutral pH (7) of the low temperature. The highest peak in low

temperature was obtained in the 2nd week. The statistically significant difference was present in the weeks of neutral pH of low temperature. So, these value ranges of low temperature supported the natural environmental conditions of temperature of the Kalar Kahar Lake.

In the case of normal room temperature, the growth of *P.bursaria* in the B-5 basal medium was reached at 747.33±30.19. It was the maximum growth obtained in the normal room temperature at the basic pH (8-9). This highest value was obtained in the 2nd week of culturing.

So, this normal room temperature (20-30) supported the natural environmental conditions of temperature of the sample site (Kalar Kahar Lake).

In the case of incubator temperature (30-40), the growth of *P.bursaria* in the B-5 Basal medium was observed. The maximum growth was in the 2nd week and it reached up-to 894.00±91.0. The highest peak was obtained in the basic pH (8-9). The temperature of natural conditions was highest at 32°C, so it is concluded that the incubator temperature (30-40) also supported the growth of *P.bursaria* like their natural environmental conditions.

In the study of Khan et al. (2011), he observed the environmental parameters of the Kalar Kahar Lake. The pH values of Kalar Kahar Lake ranged from 6.46 to 7.98. In the research experiments, three different pH were used including acidic pH (5-6), neutral pH (7) and basic pH (8-9). In the low temperature, the growth was highest in the neutral pH which reached up-to 38.33±1.76. The highest pH was obtained the 2nd week of the culturing. So, it is concluded that the neutral pH ranged within the 6.46 to 7.98 and supported the natural environmental conditions.

In the normal room temperature the highest growth was observed in the basic pH (8-9) which reached up-to 747.33±16.5. The highest growth was obtained in the 2nd week of the culturing. Also, in the incubator temperature, the highest growth was observed in the basic pH. The growth reached up-to the 894.00±91.00. The growth was highest in the 2nd week of culturing. This pH range of normal room temperature and incubators were exceeded from the natural pH range of the natural environment of Kalar Kahar Lake. But according to the study of Jafari and Alavi (2010), ciliates can grow in the pH range of 7.18-8.12. Also, according to the study of Kulaš et al. (2021), the ciliates can grow above the neutral pH. According to his study the values of pH ranged from 7.75-8.35. Ciliates can resist the basic pH and can grow in the basic medium.

In the study of Dias et al. (2008), he observed the environmental parameters of the ciliates and the values of pH ranged from 7.43-8.11. So, these studies helped to conclude that the *Paramecium bursaria* can be cultured in the basic pH because they can resist increase in basic pH.

Importance of use of different mediums for growth of *Paramecium bursaria*

Paramecium can grow in the liquid medium. These infusions have been made of animal and plant materials. These infusions will help the microorganisms like bacteria, unicellular algae and yeast to grow. These microorganisms will serve as food for *Paramecium*. The problem was that plants are sprayed with insecticides which can be lethal for *Paramecium* and can slow down their growth. Three different mediums were used in this research to culture ciliates including Hay infusion, alfalfa medium and B-5 basal medium (Sonneborn, 1970).

Basic wheat medium

In this medium boil the wheat seeds and add 60-70 seeds per1 liter of water. Allow the seed to open in the air and stand in the water for a week. This will increase the number of bacteria. Add *Paramecium* in the culture to grow. After 2 weeks the *Paramecium* will be abundant in the culture (Hyman, 1931).

In this medium, boil 6 seeds for 10 minutes and then add in the 500 ml of water. This will help bacteria to feed upon it and bacteria are the source of food for paramecia. So, basic wheat seeds help *Paramecium* to grow (Lewis & Collins, 2010).

Brewer's yeast

The Brewer's yeast increases the lifetime of the *Paramecium* culture. It contains vitamin

B. The amount of Brewer's yeast is to add ~ 0.12g /500 ml of distilled water. The amount should not cross 0.254 g in the culture otherwise the negative effect will be faced. So, it is concluded that the Brewer's yeast helps the *Paramecium* to grow fast (Lewis & Collins, 2010).

Lettuce medium

Paramecium bursaria can grow exponentially in the lettuce medium. The dried lettuce medium was sterilized at 120°C for 20 minutes and was suspended in the bottle with a wide mouth. It was then stored at 4°C. The study revealed that the cell growth density of the *P.bursaria* was 1000-1400 cells/ ml in 40-60 days. So, it is obvious that the lettuce infusion enhanced the growth of the *Paramecium bursaria* (Matsushima et al., 2022).

Hay infusion

In Hay infusion, Timothy hay was used to make the medium. Hay infusion is used as a suitable and basic medium for ciliates culturing. This infusion was useful to provide natural habitat for culturing *Paramecium*. Hay infusion was added in distilled water in 1:1 ratio and then poured in the petri dishes. The main importance of this medium was that it provided rich bacteria to ciliates, so ciliates can grow continuously. By adding wheat grains in culture media, it increases the source of food for the further enhanced growth of ciliates. Sub-cultures were regularly checked after every week (Rao & Hussain, 2012). According to (Sonneborn, 1970), Hay infusion by boiling the hay in the distilled water for 15 to 20 minutes at 120°C. After cooling it Hay infusion can be used for the stock culturing. It allows for the best isolation this infusion was best for the research experiment. So, this experimental study showed that Hay infusion plays an important role in the growth of *Paramecium bursaria*.

Alfalfa medium

To prepare this medium, one alfalfa talk is added in one liter of distilled water 1×Chalkey's solution. 10 boiled seeds were also added in the medium. In alfalfa medium, different ciliate were cultured. It is an excellent medium for growth of ciliated in large amounts, especially *Paramecium*. Alfalfa medium was formed by adding one stalk of alfalfa into 1000 ml of distilled water. The solution was boiled and after that Chalkey's solution was added into the medium. The sediments were formed by bacteria to feed upon it. Chalkey's solution was defined chemically, and has balanced salt concentration. It helps to store the medium longer for months (Hummer Jr, 1993). Although this medium was best for storage of mediums, this medium was not suitable for the growth of *Paramecium bursaria*.

B-5 Basal medium

This B-5 Basal medium was used to culture plant cells that were further used by ciliates. In this experiment the highest growth was observed in the B-5 Basal medium. This medium supports the *Paramecium bursaria* because algae grows in this medium which is eaten by *Paramecium bursaria*. So, it is concluded that the B-5 Basal medium is the best medium to culture *Paramecium bursaria* in incubator temperature (30-40) and neutral pH (7). Basic pH is also acceptable by *P.bursaria* because they are resistant to increase in pH.

Limitations

- One of the limitations of this research work was to remove algae from the samples during the process of centrifugation.
- The other limitation of our research work was that, no such growth was obtained in the alfalfa medium
- Small single cell organisms are difficult to handle and need further practice.

- Live counting of ciliates was tough and not much precise due to physiological changes and their rapid movement.
- Further research studies are required.

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